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14. ABSTRACT
We previously demonstrated that radiation therapy (RT) and hormone therapy (HT) induce tumor-specific autoantibody responses in human prostate cancer, and this grant is investigating the clinical significance of these findings. In Aim 1, the Shionogi mouse tumor model is being used to study the effect of HT and RT induced immune responses on tumor recurrence. In the past year, we have shown that HT induces autoantibody and T cell responses against the tumor antigen Poly A Binding Protein (PABP) in this model. Contrary to our hypothesis, these immune responses are associated with earlier tumor recurrence, which underscores the importance of performing analogous studies in human prostate cancer patients (Aims 2 and 3). To this end, we have established a platform for testing human T cell responses against serologically-defined tumor antigens, and we have collected large blood samples from prostate cancer patients showing treatment-induced autoantibody responses (Aim 2). We have also started to assemble cohorts of prostate cancer patients with recurrent versus non-recurrent disease at 5 years post-treatment (Aim 3). In summary, this study is progressing on schedule and is revealing unexpected results that we believe may be highly relevant to prognosis and treatment of prostate cancer.

15. SUBJECT TERMS
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W81XWH-07-1-0259 Annual Report, March 2008

PI: Brad H. Nelson, Ph.D.

Title of Project: Exploiting the Immunological Effects of Standard Treatments in Prostate Cancer

Introduction:

While much effort is being made to develop effective immune-based therapies for prostate cancer, there is little information available on whether standard treatments induce tumor-specific immune responses, which could potentially influence clinical outcomes. Radiation therapy causes inflammation associated with the expression of inflammatory cytokines, MHC molecules, B7 and other co-stimulatory molecules. Likewise, neoadjuvant hormone therapy has been shown to cause prominent T-cell infiltration of prostate tumors. Based on such findings, we asked whether radiation therapy (RT) and hormone therapy (HT), by causing tumor cell death in an inflammatory context, might induce tumor-specific immune responses in prostate cancer. Our preliminary results in the androgen-dependent murine Shionogi tumor model indicate that castration (the laboratory equivalent of HT) induces tumor-specific autoantibody responses in approximately 50% of animals. Moreover, parallel studies of human prostate cancer patients undergoing standard treatments at our institution indicate that HT and RT both induce tumor-specific autoantibody responses in up to 30% of patients, depending on the stage of disease and specific treatment. Based on these observations, we hypothesized that treatment-induced autoantibody responses in prostate cancer are accompanied by CD4+ and CD8+ T cell responses that potentially delay or prevent tumor recurrence.

This study has three specific aims:

Aim 1. To determine in the Shionogi mouse tumor model whether castration and brachytherapy induce autoantibody and T-cell responses that prevent or delay tumor recurrence.

Aim 2. To determine in human prostate cancer patients whether treatment-induced autoantibody responses are accompanied by tumor-specific CD4+ and CD8+ T-cell responses.

Aim 3. To determine whether tumor-specific autoantibody profiles differ in prostate cancer patients with recurrent versus non-recurrent disease.

Body:

Aim 1. To determine in the Shionogi mouse tumor model whether castration and brachytherapy induce autoantibody and T-cell responses that prevent or delay tumor recurrence.

We have previously shown that castration of mice bearing Shionogi tumors induces an antibody response against a ~40kd antigen in about 50% of animals (Nesslinger et. al. *Clin. Can. Res.* 13:1493). SEREX screening was performed and lead to the successful identification of the ~40kd antigen as Poly A Binding Protein (PABP). Western blotting revealed that PABP is widely expressed in various normal tissues, and is expressed at slightly higher levels in Shionogi tumors. In this regard, PABP is typical of many serologically defined human tumor

antigens, in that it exhibits a fairly widespread expression pattern. Antibodies to PABP arise in 55% of tumor-bearing mice within ~26 days of castration (Fig. 1). Interferon-gamma ELISPOT analysis revealed the presence of CD4+ and CD8+ T cell response to PABP in castrated, tumor-bearing mice. This is accompanied by dense infiltration of Shionogi tumors by CD4+ and CD8+ T cells within 1-2 weeks of castration (Fig. 2). Thus, it appears that the process of tumor regression after castration induces both T cell and antibody responses against PABP in the majority of mice.

In the Shionogi tumor model, the vast majority of castrated mice experience tumor recurrence within 2-3 weeks of castration. Contrary to our hypothesis, those mice showing antibody responses to PABP were more likely to experience tumor recurrence (Fig. 3). Moreover, tumor recurrence tended to occur more rapidly in mice with antibodies to PABP. Mice with recurrent tumors had robust systemic T cell responses to PABP, as measured by interferon-gamma ELISPOT analysis of splenocytes (Fig. 4). However, histological examination revealed that T cells were invariably restricted to the peripheral stroma of recurrent tumors and failed to penetrate the malignant epithelium (Fig. 5). Thus, treatment-induced immune responses appear to be ineffective and possibly even detrimental with respect to tumor recurrence in this model. We believe these findings may have profound implications for those human prostate cancer patients who mount tumor-associated antibody responses during treatment (Nesslinger et. al. *Clin. Can. Res.* 13:1493). It may prove effective to inhibit rather than enhance such responses. Alternatively, it may be possible to use immunomodulatory cytokines to convert these immune responses to a cytolytic, tumor-destructive phenotype. To this end, are currently performing T cell depletion experiments in the Shionogi mouse model (as originally proposed) to test the revised hypothesis that tumor-associated T cell and B cell responses may promote rather than inhibit tumor recurrence. Once these depletion experiments are completed, the results will be submitted for publication (likely by the end of 2008).

More recently, we have demonstrated that a combination of castration and irradiation of one tumor site by brachytherapy induces an abscopal effect against a second, distal tumor. Cohorts of 10 mice were implanted with two Shionogi tumors (one tumor per flank). When tumors reached 65-100 mm^2, mice were castrated to induce regression of both tumors. When tumors had fully regressed, radioactive pellets were implanted in the left flank at the site of the regressed tumor in one cohort of mice. A second cohort of mice received castration alone (i.e., without brachytherapy). Mice were monitored for tumor recurrence. Whereas non-irradiated mice experienced recurrence of both tumors with 1-3 weeks, mice receiving brachytherapy showed (a) no recurrence of the irradiated tumor, and (b) delayed recurrence of the distal, non-irradiated tumor. This latter observation is consistent with an abscopal effect. It suggests that brachytherapy (as opposed to castration alone) may promote anti-tumor immunity. Serial blood samples were collected from mice before, during and after treatment, and western blotting is underway to determine whether the prevalence or magnitude of treatment-induced antibody responses differs between the two cohorts of mice. Furthermore, splenocytes collected at the time of sacrifice will be assessed by ELISPOT for T cell responses, as described above. Finally, this spring we will test the immunomodulatory effects of Flt3 ligand in this system, as proposed.

Aim 2. To determine in human prostate cancer patients whether treatment-induced autoantibody responses are accompanied by tumor-specific CD4+ and CD8+ T-cell responses.

To investigate tumor-specific T cell responses in human prostate cancer, we are currently collecting large (200 ml) blood samples from the four prostate cancer patients who showed treatment-induced antibody responses against specific antigens that we had previously

identified by SEREX (Nesslinger et. al. *Clin. Can. Res.* 13:1493). Fortunately, all four patients are still alive and willing to provide additional blood. Additional patients will be recruited as needed. We have also established the necessary technical platforms for performing T cell assays. Specifically, we have established methods to express human tumor antigens in autologous dendritic cells by transfection of *in vitro* transcribed mRNA. We have used a MART1-specific human CD8+ T cell clone (generously provided by Dr. Cassian Yee) to show that dendritic cells transfected with MART1 mRNA can stimulate a potent MART1-specific CD8+ T cell response. Thus, we are poised to study T cell responses to SEREX-defined antigens as originally proposed.

Aim 3. To determine whether tumor-specific autoantibody profiles differ in prostate cancer patients with recurrent versus non-recurrent disease.

As proposed, we have begun collecting blood from prostate cancer patients treated approximately 5 years ago who now have recurrent (n= 9) versus non-recurrent (n= 38) disease. While we are on track to recruit 50 non-recurrent patients, the number of recurrent cases is lower than expected (a tribute to the efficacy of current treatments). Therefore, we intend to expand our network of collaborators to include Dr. Martin Gleave and others in the Greater Vancouver area, which has a five-fold larger population. We are also making preparations to create the yeast expression library for serological screening, as proposed.

Key Research Accomplishments:

The following items have been completed or are underway:

Task 1. To determine in the Shionogi mouse tumor model whether castration and brachytherapy induce autoantibody and T cell responses that prevent or delay tumor growth.

a. Obtain University of British Columbia Animal Care Committee approval ***completed***
b. Perform pilot study on 5 mice per arm including hormone therapy and brachytherapy (HT+BT), hormone therapy and sham radiation (HT+sham) and hormone therapy only (HT-only) to test the efficacy of the BT pellets (Months 1-2). ***completed***
c. Repeat experiment using 20-40 mice per arm (Months 3-7). ***in progress***
d. Perform Western blotting using the serial blood draws from the pilot and expanded study (Months 3-9). ***in progress***
e. Construct a yeast display library using Shionogi tumor mRNA (Months 1-3). ***completed, used phage library instead***
f. Screen the yeast display library using serum from mice that show a treatment-induced immune response to identify the ~40 kDa tumor-specific antigen identified in the preliminary studies (Months 4-9). ***completed***
g. Perform the peptide library screen to characterize T cell epitopes (Months 9-10). ***completed, unsuccessful, but no longer considered necessary***
h. Perform T cell assays including lymphoproliferative assays, intracellular cytokine staining and examination of tumor infiltrating lymphocytes (Months 11-14). ***in progress***
i. Perform CD19+, CD8+ and CD4+ depletion studies including production of the monoclonal antibodies, performing the depletion studies in 4 groups of 5 mice and analyzing the serum by Western blot (Months 15-20). ***monoclonal antibodies are currently being produced for depletion experiments Summer 2008***

j. Perform the Flt3 ligand studies on groups of 5 mice, including analysis of B and T cell responses by Western blot, proliferative assays and intracellular cytokine staining (Months 20-26). ***planned for Spring 2008***
k. Perform the dendritic cell vaccination studies, including preparation of the dendritic cells, vaccinating 10 mice in conjunction with the HT+BT regimen (5 with relevant and 5 with irrelevant peptide), Western blot analysis of serial blood draws and T cell assays (Months 27-33). ***planned for 2008/2009***

Task 2. To determine in human prostate cancer patients whether treatment-induced autoantibody responses are accompanied by tumor-specific CD4+ and CD8+ T cell responses.

a. Construct a yeast phage display library from three prostate cancer cell lines: LNCaP, DU145 and PC3 (Months 1-3). ***planned for 2008***
b. Screen the remaining 79 patients using the Western blot assay as additional post-treatment samples become available to identify treatment-induced immune responses (Months 1-6). ***deferred while we complete our analysis of the initial patient cohort***
c. Screen the yeast phage display library with serum from the patients identified by Western as having treatment-induced responses (Months 6-9). ***ditto***
d. Perform T cell assays using the antigens already identified plus additional antigens identified using the yeast library screening, including dendritic cell transfection, intracellular cytokine staining, and T cell proliferation assay (Months 10-16) ***platform for performing T cell assays has been successfully established; assays will be performed in 2008/2009***
e. Monitor the longevity of treatment-induced immune responses in the 16 patients already identified plus any additional patients identified above using Western blot, antigen array and T cell assays where applicable (Months 30-36) ***planned for 2009/2010***

Task 3. To determine whether tumor-specific autoantibody profiles differ in prostate cancer patients with recurrent versus non-recurrent disease.

a. Obtain approval from the BC Cancer Agency Research Ethics Board to collect blood from recurrent versus non-recurrent prostate cancer patients (Months 1-2). ***completed***
b. Accrue 50 recurrent and 50 non-recurrent prostate cancer patients (Months 3-12). ***in progress, expanding recruitment network to Vancouver***
c. Screen the prostate cancer yeast display library with 5 recurrent and 5 non-recurrent screening pools (Months 17-21) ***planned for 2009***
d. Construct an antigen array with the antigens cloned above plus previously cloned antigens and screen the array with the screening pools and the validation pools (Months 22-26). ***planned for 2009/2010***
e. Perform data and statistical analysis (Months 27-28).***planned for 2010***
f. Screen the array using the 134 patient cohort to assess the correlation to clinical outcomes (Months 30-36). ***planned for 2010***

Reportable Outcomes:

Peer-reviewed manuscripts:

Nesslinger, N.J., Sahota, R.A., Stone, B., Johnson, K., Chima, N., Bishop, D., Rennie, P.S., Pai, H., Ludgate, C., **Nelson, B.H.** 2007. Standard treatments induce antigen-specific immune responses in prostate cancer. *Clinical Cancer Research.* Mar 1; 13(5):1493-502. PMID: 17332294.

Nesslinger, N.J., Pai, H.H., Ludgate, C.M., **Nelson, B.H.** 2008. Exploring the effects of standard treatments on the immune response to prostate cancer. *Methods of cancer diagnosis, therapy, and prognosis*. *Springer, Vol. 2.*

Conference proceedings:

Hormone therapy induces antigen-specific antibody and T cell responses in the Shionogi mouse tumour model. Sara Hahn, Nancy Nesslinger, Rob Drapala, Mary Bowden, Paul Rennie, Howard Pai, Charles Ludgate, **Brad H. Nelson**. Annual Meeting of the American Association for Cancer Research, San Diego, CA, April 2008.

Invited presentations:

Standard Treatments Induce Antigen-Specific Immune Responses in Prostate Cancer. **Brad H. Nelson.** Prostate Cancer Research Foundation of Canada Retreat, Orangeville, ON, January 2007.

Toward Predictive and Personalized Immunotherapy of Cancer. **Brad H. Nelson**. Dept. of Immunology, University of Minnesota, Minneapolis MN, April 2007.

Toward Predictive and Personalized Immunotherapy of Cancer. **Brad H. Nelson**. UBC Medical Genetics, Vancouver BC, May 2007.

Toward Predictive and Personalized Immunotherapy of Cancer. **Brad H. Nelson**. Canadian Melanoma Conference, Banff AB, Oct 2007.

Toward Predictive and Personalized Immunotherapy of Cancer. **Brad H. Nelson**. UBC Immunology Department Seminar, Vancouver BC, Oct. 2007.

Tracking and Manipulating the Immune Response to Cancer. **Brad H. Nelson**. City of Hope Medical Center, Duarte CA, Mar. 2008.

Collaborations:

We have established a collaboration with Dr. James Gulley at the NCI to analyze tumor-specific immunity in prostate cancer patients who participated in a Phase I clinical trial involving a poxviral vaccine encoding prostate-specific antigen (PSA), administered with GM-CSF and IL-2:

Gulley, J.L., Arlen, P.M., Bastian, A., Morin, S., Marte, J., Beetham, P., Tsang, K.Y., Yokokawa, J., Hodge, J.W., Menard, C., Camphausen, K., Coleman, C.N., Sullivan, F., Steinberg, S.M., Schlom, J., Dahut, W. 2005 Combining a Recombinant Cancer Vaccine with Standard Definitive Radiotherapy in Patients with Localized Prostate Cancer *Clinical Cancer Research.* May 1; 11: 3353-3362

Career advancement:

Sara Hahn has performed the majority of the mouse experiments in Aim 1, which have been submitted for her Masters thesis at the University of Victoria. The results are also expected to be submitted for publication by the end of 2008.

Conclusions:

Both the murine and human phases of this project are proceeding on schedule. No major obstacles have been encountered apart from some difficulty finding sufficient numbers of patients with recurrent prostate cancer 5 years post treatment. This will be addressed by expanding our network of collaborators to include oncologists in Vancouver BC, which has a five-fold larger population.

Importantly, the murine studies have lead to the unexpected observation that castration-induced antibody responses are associated with more prevalent and rapid tumor recurrence. This may be of profound clinical importance, as ~30% of human prostate cancer patients show treatment-associated antibody responses. It may prove effective to inhibit such responses, or convert them to a cytolytic, tumor-destructive phenotype. The Shionogi model provides a useful experimental system to investigate these important issues.

References:

None.

Appendices:

See accompanying Figures 1-5.

Annual Report Appendix, March 2008

PI: Brad H. Nelson, Ph.D.

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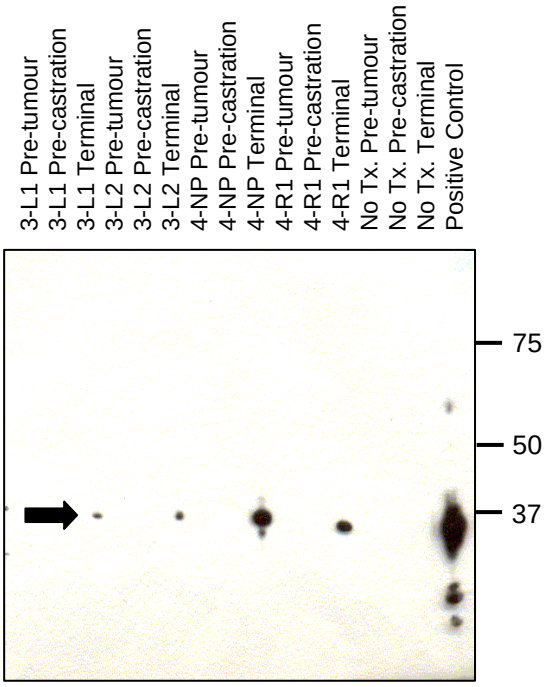

Figure 1. Castration induced an antibody response against PABPN1 in a large proportion of mice. Purified**,** soluble, *E. coli* recombinant PABPN1 (10 µg) was probed with pre-tumour, pre-castration, and terminal serum samples obtained from castrated mice 3-L1, 3-L2, 4-NP, 4-R1, and a tumour-bearing, non-castrated (No Tx.) mouse. 3-L1 and 3-L2 were sacrificed on day 28 post-castration and 4-NP and 4-R1 were sacrificed on day 33 post-castration. The arrow shows the PABPN1 seroreactive band.

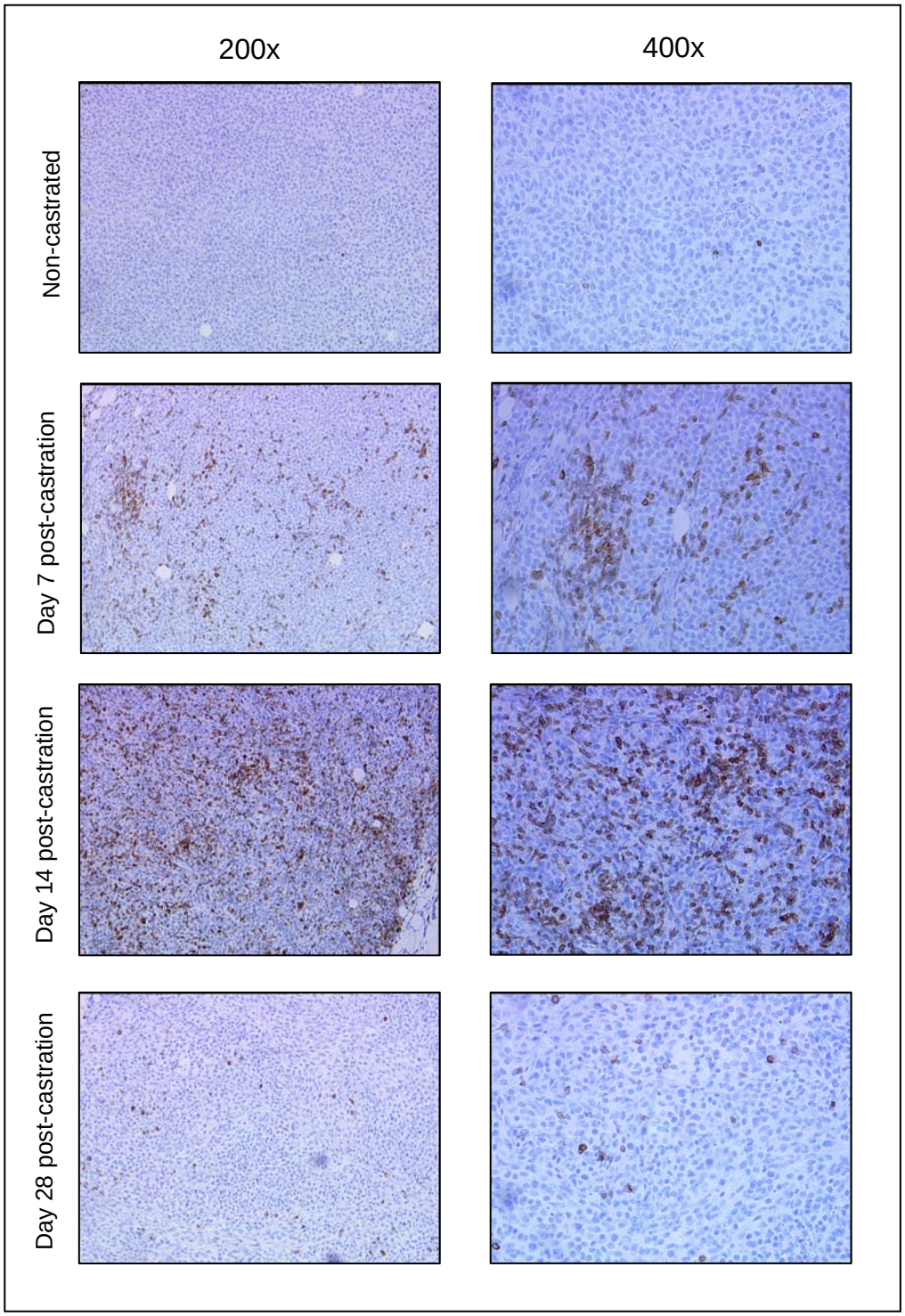

Figure 2. Anti-CD3 staining of Shionogi tumours at specific time points following castration showed dense infiltration of CD3+ T cells between 7-14 days post-castration. By 28 days post-castration, when most of the tumours had recurred, CD3+ T cells were sparse. The tumours from non-castrated mice were essentially void of CD3+ T cells.

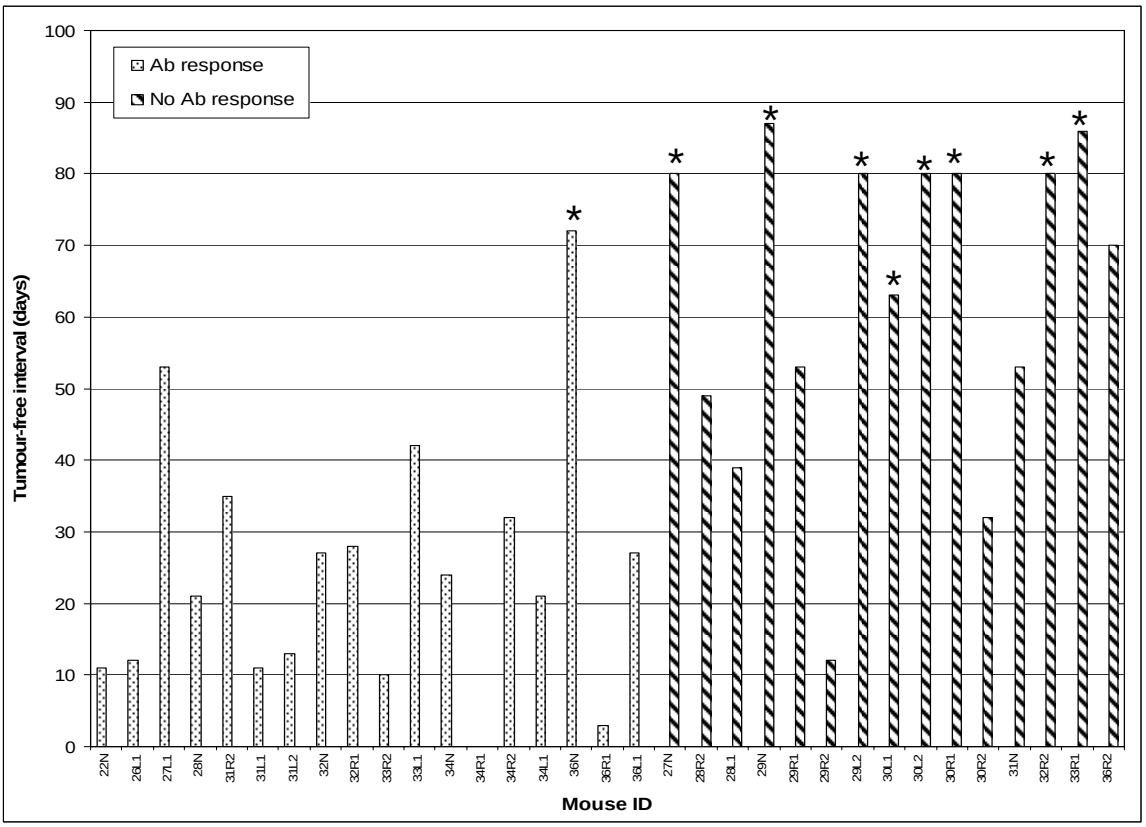

Figure 3. The majority of mice that did not have a PABPN1 antibody (Ab) response had a longer tumour-free interval compared to those mice that did have a PABPN1 antibody response. The * indicates that for these mice, the tumour-free interval was not determined, as the mice were sacrificed on the indicated day for the purpose of immunological analysis.

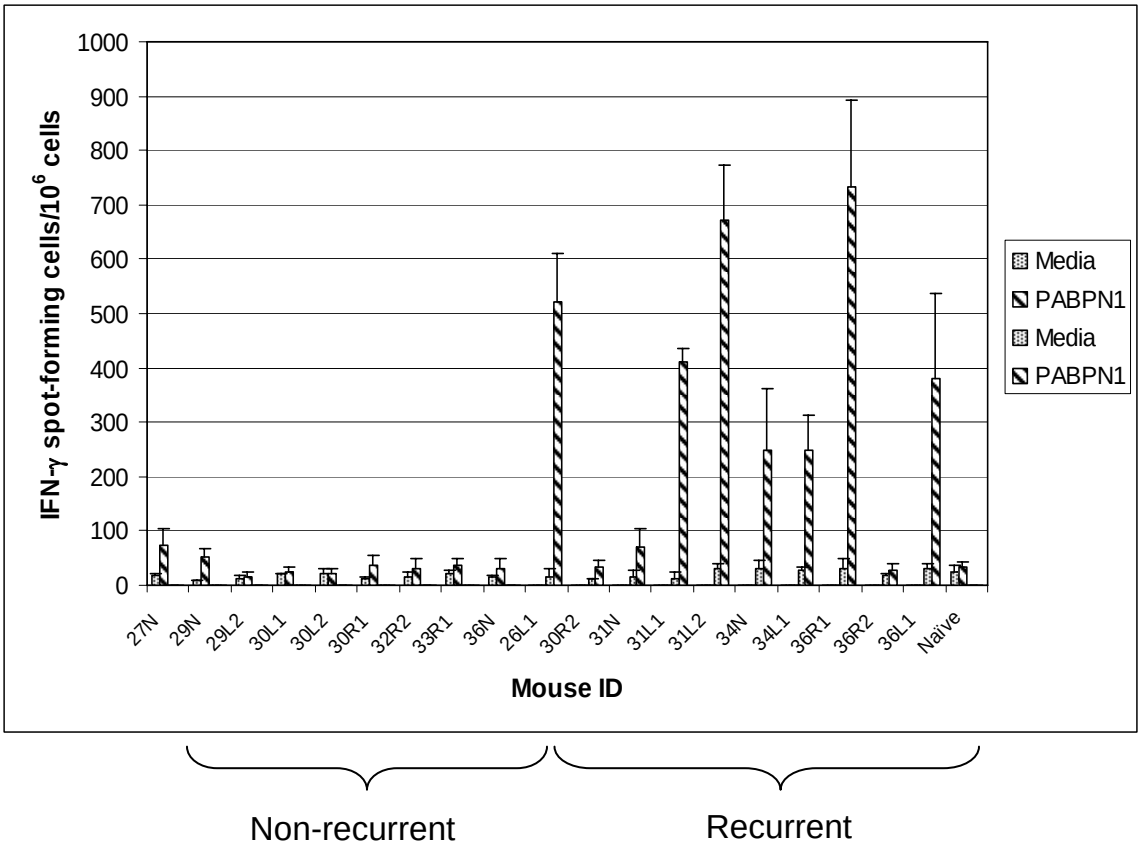

Figure 4. Castrated mice with recurrent tumours generally had a larger PABPN1-specific T cell response compared to those mice that remained tumour-free for the duration of the experiment. For each mouse shown on the graph, fresh splenocytes were used in the ELISPOT assay, which was run in triplicate, with the values being averaged and the standard deviation calculated. The splenocytes were stimulated with media as a negative control.

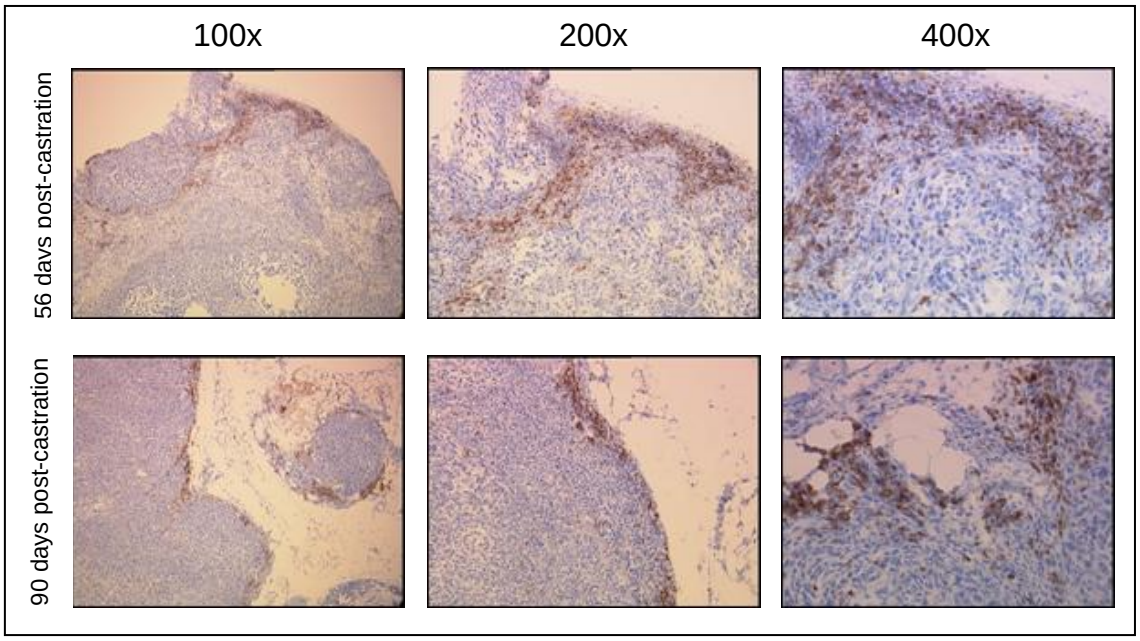

Figure 5. Representative recurrent tumours stained with anti-CD3 from one mouse sacrificed on day 56 post-castration and one mouse sacrificed on day 90 post-castration show that the CD3+ T cells are mainly confined to the periphery of the tumours and surrounding stroma.